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RESEARCH ARTICLE

**Meirav Bar-Shimon · Hila Yehuda · Lea Cohen
Batia Weiss · Alexsandra Kobeshnikov · Avinoam Daus
Martin Goldway · Michael Wisniewski · Samir Droby**

Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent *Candida oleophila*

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Abstract The yeast *Candida oleophila*, the base of the commercial product Aspire, is recommended for the control of postharvest decay of citrus and pome fruit. Competition for nutrients and space is believed to be the major mode of action. Involvement of fungal cell wall-degrading enzymes is also suggested to play a role in the mechanism of action of yeast antagonists. The present study showed that the yeast *C. oleophila* is capable of producing and secreting various cell wall-degrading enzymes, including exo-β-1,3-glucanase, chitinase and protease. Exo-β-1,3-glucanase and chitinase were produced and maximized in the early stages of growth, whereas protease reached a maximum level only after 6–8 days. Production of exo-β-1,3-glucanase, chitinase and protease was stimulated by the presence of cell wall fragments of *Penicillium digitatum* in the growth medium, in addition to glucose. This study also provided evidence that *C. oleophila* is capable of secreting exo-β-1,3-glucanase into the wounded surface of grapefruit. The role of exo-β-1,3-glucanase (*CoEXG1*) in the biocontrol activity of *C. oleophila* was tested using *CoEXG1*-knockouts and double-*CoEXG1* over-producing transformants. In vitro bioassays showed that wild-type *C. oleophila* and exo-β-1,3-glucanase over-expressing transformants had similar inhibitory effects on spore germination and germ-tube elongation; and both were more inhibitory to the fungus than the knockout transformant. In experiments conducted on fruit to test the biocontrol activity against infection by *P. digitatum*, no significant difference in inhibition was observed between transformants and untransformed *C. oleophila* cells at the high concentrations of cells used, whereas at a lower concentration of yeast cells the knockout transformants appeared to be less effective.

Keywords Yeast · *Candida oleophila* · Biological control · Postharvest · Lytic enzymes

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M. Bar-Shimon · L. Cohen · B. Weiss · A. Kobeshnikov
A. Daus · S. Droby (✉)
Department of Postharvest Science, A.R.O.,
The Volcani Center, P.O. Box 6,
50250 Bet Dagan, Israel
E-mail: samird@volcani.agri.gov.il
Tel.: +972-3-9683615
Fax: +972-3-9683856

H. Yehuda · M. Goldway
Department of Molecular Genetics,
MIGAL Galilee Technological Center,
P.O. Box 831, Kiryat Shmona, Israel

M. Wisniewski
Appalachian Fruit Research Station,
USDA-ARS, 45 Wiltshire Road,
Kearneysville, WV 25430, USA

Introduction

The development of biological control methods, as an alternative to chemical fungicides in the control of postharvest diseases, has been on a fast-track in recent years. Several yeasts and bacteria have been shown to protect against a number of postharvest pathogens on a variety of harvested commodities (Droby et al. 1991; Janisiewicz et al. 1994; Chand-Goyal and Spots 1996; El-Ghaouth et al. 1998; Ippolito et al. 2000; Kurtzmann and Droby 2002). Currently, four antagonistic micro-organisms—two yeasts (*Candida oleophila*, *Cryptococcus albidus*) and two strains of the bacterium *Pseudomonas syringae*—are commercially available under the trade names Aspire (Ecogen, Langhorn, Pa., USA), YieldPlus (Anchor Yeast, Cape Town, South Africa) and BIO-SAVE-110 and -111 (EcoScience, Orlando, Fla., USA), respectively. The main problem that prevents wide-spread application of these products is their reduced efficacy and inconsistency under commercial conditions (Droby et al. 1998). Enhancing the activity of biocontrol agents is an important factor in ensuring their success in controlling fruit diseases and in their ultimate acceptance in the commercial management of diseases. When considering how to improve the performance of a natural biocontrol agent and hence to develop it as a

reliable commercial product, it is advantageous first to characterize its mechanism(s) of action.

Antagonistic yeasts have been selected mainly for their ability to colonize and grow rapidly in surface wounds and subsequently out-compete the pathogen for nutrients and space (Droby et al. 2000). Competition for nutrients and space is believed to be the major mode of action of antagonistic yeasts (Droby et al. 1989; Wisniewski et al. 1991; Droby and Chalutz 1994; El-Ghaouth et al. 1998). In addition, several studies have shown that antagonistic yeasts are capable of inducing resistance mechanisms in the host tissue (Rodov et al. 1994; Fajardo et al. 1998; Porat et al. 1999; Droby et al. 2002).

There is growing evidence to support the possible involvement of cell wall-degrading enzymes in the action of yeast antagonists. Wisniewski et al. (1991) showed that the yeast *Pichia guilliermondii* produced and secreted high levels of exo-β-1,3-glucanase and chitinase when cultured on various carbon sources or on the cell walls of fungal pathogens. The ability of the yeast to produce exo-β-1,3-glucanase and chitinase is hypothesized to be associated with the firm attachment of the yeast cells to fungal hyphae and the partial degradation of fungal mycelia (Wisniewski et al. 1991). Grevesse et al. (2003) suggested the involvement of exo-β-1,3-glucanase in the biocontrol activity of the yeast *P. anomala* against *Botrytis cinerea* on apples. They found that purified exo-β-1,3-glucanase from yeast culture filtrates showed an inhibitory effect in vitro on the germination and germ tube growth of *B. cinerea*. Also, the addition of *B. cinerea* cell walls to a suspension of *P. anomala* stimulated in situ exo-β-1,3-glucanase activity. In experiments with *Aureobasidium pullulans*, a yeast-like antagonist of postharvest pathogens, exo-β-1,3-glucanase activity was detected both in vitro and in apple wounds (Castoria et al. 2001). In addition, the exo-β-1,3-glucanase activities of two different antagonistic yeasts, *Rhodotorula glutinis* (LS-11) and *C. laurentii* (LS-28), were higher in the culture filtrate of the more effective antagonist (Castoria et al. 1997).

The yeast *Candida oleophila* Montrocher (strain 182) is the basis of the commercial product Aspire, which is commercially used against the decay of citrus and apple fruit. Recently, the gene *CoEXG1*, which encodes a secreted exo-β-1,3-glucanase was isolated from *C. oleophila* (Segal et al. 2002). The role of exo-β-1,3-glucanase in the biocontrol activity of *C. oleophila* was investigated by generating *C. oleophila CoEXG1*-knockouts and double-*CoEXG1* transformants (Yehuda et al. 2001, 2003). It was found that *CoEXG1*-knockout transformants lost their ability to secrete exo-β-1,3-glucanase in to the growing medium, while the double-*CoEXG1* transformants secreted approximately twice as much exo-β-1,3-glucanase as the untransformed *C. oleophila*. In contrast, the biocontrol activity of *CoEXG1*-knockout and double-*CoEXG1* transformants against *Penicillium digitatum* on kumquat fruits did not differ from that of untransformed *C. oleophila* (Yehuda et al. 2003). This suggested that, under those experimental conditions, the exo-β-1,3-glucanase secreted by *C. oleophila* is not essential in the biocontrol activity against *P. digitatum* on kumquats and it may react in an additive or synergistic manner with other genes or be active in biocontrol under conditions different from those examined (Yehuda et al. 2003).

Therefore, the objectives of this study were: (1) to characterize the capability of the yeast *C. oleophila* to produce and secrete other fungal cell wall-lytic enzymes (e.g., chitinase, proteases) in culture and in fruit surface wounds, (2) to study the effect of various carbon sources on the production of these enzymes, (3) to test ,in vitro, the effect of secreted enzymes of transformed and untransformed *C. oleophila* on spore germination and germ-tube elongation of *P. digitatum* and (4) to determine the antagonistic activity of transformants against *P. digitatum* infection on grapefruit.

Materials and methods

Yeast and fungal cultures

The yeast *C. oleophila* Montrocher (strain 182) was isolated from the surface of tomato fruits (Wilson et al. 1993). The yeast culture was maintained at −18 °C on silica beads coated with skim milk; and cultures were grown on nutrient yeast dextrose agar (NYDA) containing 8 g of nutrient broth, 5 g of yeast extract, 10 g of D-glucose and 20 g of agar in 1 l of distilled water. Liquid cultures of the yeast were grown in 250-ml Erlenmeyer flasks containing NYDB and incubated at 25 °C for up to 8 days on an orbital shaker. A culture of *P. digitatum* (Pers. Fr.) Sacc. was isolated from decayed citrus fruits, stored on potato dextrose agar (PDA; Difco Laboratories, Detroit, Mich., USA) slants at 4 °C, and grown on PDA plates for 1 week at 25 °C. Spore suspensions were prepared by removing spores from the sporulating edges of a 2- to 3-week-old culture with a bacteriological loop and suspending them in sterile distilled water. The spore concentration was adjusted to 5×10^4 spores ml^{-1} with a hemacytometer.

The construction of the double-*CoEXG1* and *CoEXG1*-knockout *C. oleophila* transformants was described by Yehuda et al. (2001, 2003).

Enzyme assays

For enzyme assays, aliquots of 10 ml of growth medium were aseptically withdrawn at various intervals during the growth period, centrifuged at 6,136 *g* for 10 min to remove yeast cells, filtered through a Millipore membrane (0.2 μm) and kept at −20 °C until used. The pelleted yeast cells were suspended in sterile distilled water to the initial volume; and the concentration of yeast cells was calculated according to a calibration curve based on the measurement of absorbance at 600 nm (A_{600}).

Exo-β-1,3-glucanase activity assay

This enzyme assay was carried out by adding 0.2 ml of 4 mM 4-methylumbelliferyl-β-D-glucoside (MUG; Sigma Chemical Co., St. Louis Mo., USA) in citrate phosphate buffer (12 mM citric acid, 50 mM NaH_2PO_4, pH 5.0) to 0.6 ml of *C. oleophila* culture filtrate. The reaction mixture was incubated at 50 °C for 30 min and stopped by the addition of 0.2 ml of cold trichloroacetic acid (TCA; 25%). Aliquots of 0.2 ml of the reaction mixture were diluted 20-fold in glycine bicarbonate buffer (133 mM glycine, 83 mM

Na_2CO_3, pH 10.7) and the liberated 4-methylumbelliferone was immediately measured with a microplate fluorescence reader (BIO-TEK Instruments, USA; excitation at 350 nm, emission at 440 nm). Exo-glucanase activity was determined by comparison with a calibration curve based on the release of 4-methylumbelliferone with one unit of activity being defined as the number of nanomoles of 4-methylumbelliferone released per minute per milliliter.

Chitinase activity assay

The reaction mixture was made up by adding 0.2 ml of a solution (2 mg ml^{-1}) of carboxymethyl-chitin remazol brilliant violet (Loewe Biochemica, Germany) in citrate phosphate buffer to 0.6 ml of the yeast culture filtrate; and the mixture was incubated for 1 h at 50 °C. The reaction was terminated by the addition of 0.2 ml of hydrochloric acid and the mixture was immediately cooled on ice for 10 min. Non-degraded substrate was removed by centrifugation at 9,391 *g* for 5 min and the amount of degraded substrate was measured in the supernatant by A_{550}. Enzyme activity was expressed as ΔA_{550} ml^{-1} min^{-1}.

Protease activity assay

The reaction mixture consisted of 0.2 ml of 1% azoalbumin (Sigma Chemical Co., St. Louis, Mo., USA) in 100 mM sodium acetate (pH 5.0) and 0.6 ml of culture filtrate. Following incubation for 1 h at 30 °C, the reaction was stopped by the addition of 0.2 ml of cold TCA (50%). Incubation was then continued for 1 h at 4 °C to allow for precipitation of the non-hydrolyzed azoalbumin, which was then removed by centrifugation at 9,391 *g* for 10 min. The A_{366} of the supernatant was measured and enzyme activity was expressed as ΔA_{366} ml^{-1} min^{-1}.

Partial purification of enzyme preparations

Culture filtrate of *C. oleophila* was prepared as described above using approx. 4 l of 7-day-old culture. Following filtration through a Millipore membrane (0.2 μm), proteins in the supernatant fluid were precipitated with $(NH_4)_2SO_4$ (approx. 80% saturation) on ice. The precipitate was recovered by centrifugation at 16,260 *g* for 30 min, dissolved in 0.1 M phosphate buffer (pH 7.2) and then dialyzed three times against 5 l of distilled water at 4 °C overnight. The protein solution was concentrated in a roto-evaporator (Rotovac, Switzerland) from 50 ml to 1 ml. The protein concentration was determined according to Bradford (1976), with bovine serum albumin (Sigma Chemical Co., Israel) as the standard.

Activity gel assays

Partially purified protein extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), with 10% or 15% polyacrylamide in the separating gel.

The activities of exo-β-1,3-glucanase and chitinase on the gel were determined following the removal of SDS by washing the gel four times (30 min each) with freshly prepared casein buffer (containing 40 mM Tris-HCl, pH 8.5, 1% casein, 2 mM EDTA, 0.2% sodium azide). Then, the gel was washed twice for 15 min with 50 mM sodium acetate buffer (pH 5.0). An overlaying gel was prepared by dissolving 50 mg of a low-melting agarose (FMC Bioproduct, Rockland, Me., USA) with 2–4 mg of MUG (substrate for exo-β-1,3-glucanase) or 4-methylumbelliferyl-β-1,4-di-acetylchitobiose (substrate for chitinase) in 5 ml of heated 50 mM sodium acetate buffer (pH 5.0). The overlaying gel was cast onto pre-washed PAGE gel. The gel assembly was incubated at 37 °C for 30 min and the chitinase and exo-glucanase activities were visualized under UV light at 254 nm.

Protease activity was determined on 10% SDS-PAGE containing 0.1% gelatin. After electrophoresis, the SDS was removed by washing the gel twice for 40 min with 2.5% Triton-X 100 in 10 mM Tris-HCl (pH 7.5) and then incubating it overnight in 50 mM Tris-HCl (pH 7.5) at 37 °C. The gel was stained with 0.1% amido black in 30% methyl hydroxide, 10% glacial acetic acid for 1 h, followed by destaining with a mixture of 30% methyl hydroxide, 10% glacial acetic acid, until a clear band appeared on the dark background (about 3–4 h).

Preparation of *P. digitatum* cell walls

P. digitatum was grown in 500 ml of potato dextrose broth (PDB; Difco Laboratories) seeded with 1 ml of fungal spore suspension for 6 days at 25 °C on a rotary shaker. The mycelium was collected by filtration on a Whatman filter paper (no. 1), washed three times with distilled water and homogenized in 0.1 M phosphate buffer (pH 7.2) using a Tissuemizer homogenizer (PCU Drehzahlregler; Kinematica, Germany) for 2 min; and the homogenates were kept at −20 °C overnight. The frozen homogenates were then thawed and homogenized again, as described above, to break-up the pellet. The homogenized fungal mycelium (approx. 20 ml) was transferred to an homogenizer flask (Braun, Georghkobold, Germany) and 6 g of glass beads (425–600 μm; Sigma) were added. Following 2–3 min of homogenization, the samples were kept at 4 °C for 10–15 min to allow the glass beads to settle. The liquid was then collected and centrifuged for 2 min at 1,625 *g*, the supernatant was discarded and the pellet was resuspended in water and centrifuged six times to obtain a clear supernatant. These preparations were used in the experiments that aimed to test the effects of various carbon sources on lytic enzyme production.

Effect of carbon source on lytic enzyme production

C. oleophila was cultured in a minimal salts medium (Lilly and Barnett 1951) containing: 7 mM KH_2PO_4, 10 mM L-aspargine, 20 mM $MnSO_4$, 0.3 mM thiamin, 54 mM $ZnSO_4$, 0.65 M $FeSO_4$, 2 mM $MgSO_4 \cdot 7H_2O$ and 0.4 mM biotin, with 1% glucose and/or *P. digitatum* cell wall fragments as the sole carbon source. Three separate 100-ml Erlenmeyer flasks containing 50 ml of culture medium were incubated with *C. oleophila* (10^7 cells ml^{-1}) on a rotary shaker (200 rpm) at 25 °C. Aliquots of 5 ml of yeast culture were withdrawn aseptically from each flask after 24, 48, 72, 96 and 120 h and centrifuged at 6,136 *g* for 15 min to remove yeast cells. Cell-free culture fluid was filtered through a Millipore membrane (0.2 μm) and kept at −20 °C until used. The pelleted yeast cells from each sample were suspended in a volume of sterile distilled water equal to that of the culture fluid and the concentration of yeast colony-forming units (CFU) was determined by plating 10-μl aliquots of serial dilutions on PDA plates.

Detection of exo-β-1,3-glucanase activity on surface wounds

Grapefruits were obtained from a local orchard and kept at 11 °C until used. The fruits were thoroughly rinsed with water and wounded (2–3 mm width) with a dissecting needle at six sites around their bottom end. Aliquots of 30 μl of *C. oleophila* cell suspension (10^8 cells ml^{-1}) were placed into each wound; and sterile distilled water was used for the controls. The treated wound sites were removed with a 0.5-mm diameter cork borer, immediately after treatment, and at 12, 24, 48 and 72 h; and the tissues were homogenized in 5 ml of 50 mM sodium acetate buffer, pH 5.0. The homogenates were shaken on an orbital shaker (Rotamix RM1; ELMI, Latvia) for 24 h at 4 °C in order to extract proteins from the tissue. Samples were centrifuged for 1 h at 14,674 *g* at 4 °C and the supernatant was collected and kept at −20 °C, pending assay. Three fruits, each with six wound

sites, were tested each day. Each fruit served as one replicate, which consisted of six wounds; and the experiment was conducted three times, with similar results.

Inhibitory activity assay in vitro

Constant volumes (10 µl) of *P. digitatum* spore suspension in 50% PDB (2×10^5 spores ml^{-1}) were mixed in sterile Eppendorf tubes with constant volumes (90 µl) of enzyme solutions (containing successively increasing concentrations of proteins) and incubated at 25 °C for 16–20 h. The control contained either sterile water or autoclaved enzyme solution. The number of germinating and non-germinated spores was determined in three different microscope fields containing at least 30 spores each; and the length of the germ tubes in those fields was measured and averaged, using a micrometric scale fitted in the microscope.

Biocontrol activity assay

Cultures of wild-type *C. oleophila* (strain 182) and transformants containing a double (T-201) or a knockout (T-789) of the glucanase gene were grown in NYDB for 24–48 h. Cells were collected from the growth medium by centrifugation at 6,136 *g* for 10 min. The yeast cells were resuspended in sterile distilled water and the concentration was determined by measuring the A_{600}. Grapefruits were wounded (2–3 mm width, depth) with a dissecting needle at three sites around the bottom end. Aliquots of 30 µl of *C. oleophila* (wild-type or transformant) cell suspensions at various concentrations (10^6–10^8 cells ml^{-1}) were placed in each wound; and sterile distilled water was used for the controls. The treated wounds were allowed to air-dry and were then inoculated with 30 µl of an aqueous suspension of *P. digitatum* spores (10^4 cells ml^{-1}). Fruits were kept under moist conditions for 5 days and the percentage of infected wounds was determined.

Results

Detection of extracellular lytic enzymes secreted by *C. oleophila*

Exo-β-1,3-glucanase activity of *C. oleophila* reached a maximum after 4 days of growth in NYDB medium; and the secretion level remained the same for up to 8 days (Fig. 1A). Chitinase activity secreted by *C. oleophila* reached its maximum level after 24 h of growth and started to decrease only after day 3 (Fig. 1B). In contrast, protease secreted by *C. oleophila* exhibited low activity during the initial days of growth and reached its maximum level only after 6–8 days (Fig. 1C). Enzymatic activities were detected on gels in order to estimate the molecular mass of the glucanase, chitinase and protease secreted by *C. oleophila*. For this purpose, partially purified culture filtrate was used. Each enzyme activity was determined in a different gel with a specific protocol (see the Materials and methods). Each gel used a molecular weight marker and the apparent mass of the enzyme was estimated according to the marker. Exo-β-1,3-glucanase activity was observed as a single band with a molecular mass of about 30 kDa (Fig. 2A), chitinase activity as a band with a molecular mass of 200 kDa (Fig. 2B) and protease activity as a band with a molecular mass of 60 kDa (Fig. 2C).

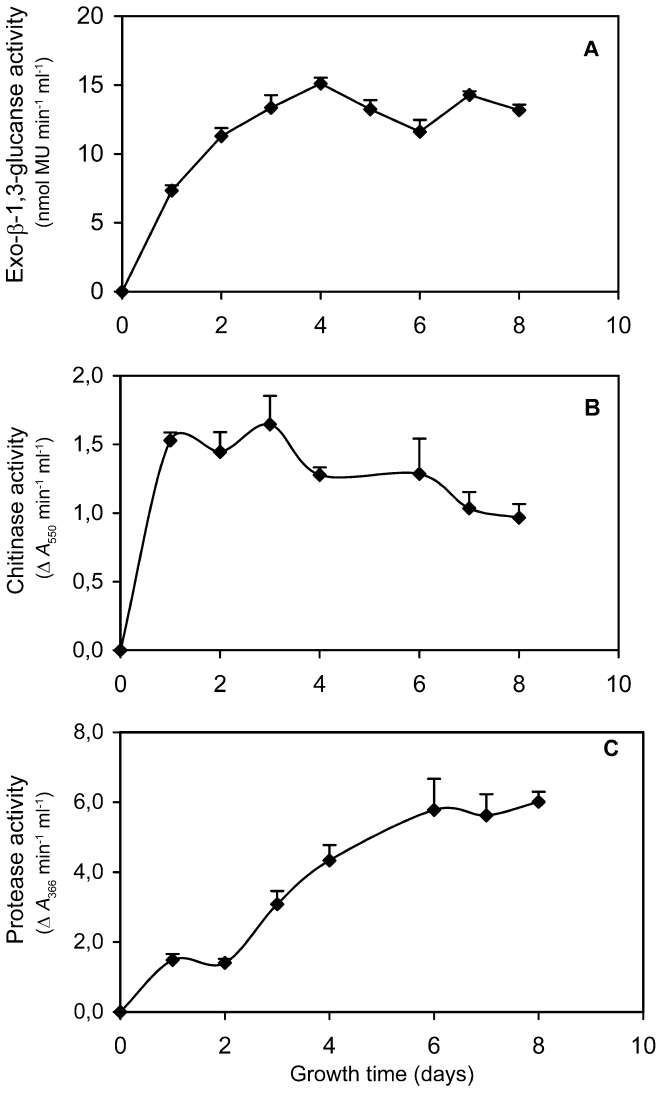

Fig. 1A–C Extracellular lytic enzyme activities of *Candida oleophila*, indicated by the time-course of enzyme secretion into the growth medium (nutrient yeast dextrose broth). **A** Exo-β-1,3-glucanase activity was expressed as nanomoles of 4-methylumbelliferone (*MU*) released from 1 ml of 4-methylumbelliferyl β-D-glucoside (MUG) in 1 min. **B** Chitinase activity was measured as an increase in the absorbance (A_{550}) of 1 ml of degraded carboxymethyl-chitin remazol brilliant violet (CM-chitin RBV) in 1 min. **C** Protease activity was measured as an increase in the A_{366} of 1 ml of azoalbumin during hydrolysis for 1 min. Bars indicate the standard error of the mean. The data from two experiments with three replicates were averaged

Effect of carbon sources on lytic enzymes production

The level of exo-β-1,3-glucanase activity detected in growth medium supplemented with *P. digitatum* cell wall fragments as a sole carbon source was very low, as compared with that in growth medium containing glucose. The former increased slightly after 24 h and remained constant for up to 5 days (Fig. 3A). In contrast, the combination of glucose and *P. digitatum* cell wall fragments in the growth medium induced the highest level of exo-β-1,3-glucanase activity, as compared with

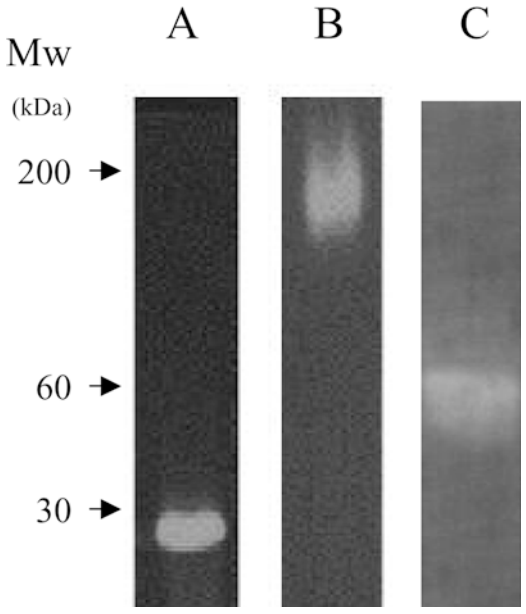

Fig. 2A–C Activity gels of extracellular lytic enzymes secreted by *C. oleophila*, showing detection of lytic enzyme activity on activity gel after SDS-PAGE. **A** Exo-β-1,3-glucanase activity was detected on 12% polyacrylamide, overlaid with agarose containing 0.05% MUG. There was 10 µg protein lane^{-1}. **B** Chitinase activity was detected on 10% polyacrylamide, overlaid with agarose containing 0.1% 4-methylumbelliferyl β-D-*N*,*N*-diacetylchitobioside. There was 120 µg protein lane^{-1}. Glucanase and chitinase activities were detected under UV light at 254 nm. **C** Protease activity was detected on 10% polyacrylamide containing 0.1% gelatin after staining with amido black. There was 40 µg protein lane^{-1}. *Mw* Molecular weight

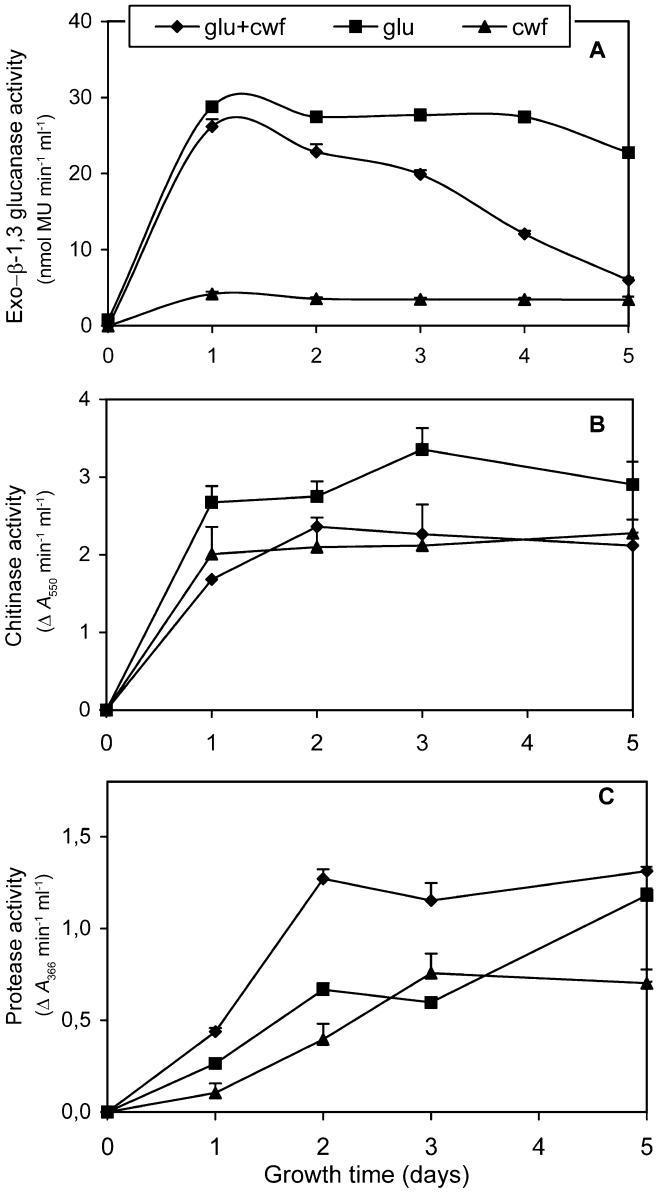

Fig. 3A–C Effect of carbon source on extracellular lytic enzymes secreted by *C. oleophila*, indicated by the time-course of lytic enzyme secretion by *C. oleophila* (strain 182) in minimal medium containing salts and either 1% glucose (*glu*) or *Penicillium digitatum* cell wall fragments (*cwf*) or both. **A** Exo-β-1,3-glucanase activity was expressed as nanomoles of 4-methylumbelliferone released from 1 ml of MUG in 1 min. **B** Chitinase activity was measured as an increase in the absorbance of 1 ml of degraded CM-chitin RBV in 1 min. **C** Protease activity was measured as the increase in the absorbance of 1 ml of azoalbumin during hydrolysis for 1 min. *Bars* represent the standard error of the mean. Each treatment consisted of three replicates and each experiment was repeated three times

that induced by glucose alone, with a significant difference that persisted from day 2 until the last day of growth.

Chitinase and protease activity, however, were not influenced significantly by either glucose or cell wall fragments as the sole carbon source (Fig. 3B, C). Similar increases in chitinase activity were measured after 24 h of growth, regardless of the carbon source, and the level remained constant with time. Higher chitinase activity was measured in medium containing cell wall fragments and glucose. In the case of protease, higher levels of activity were detected in the medium supplemented with both glucose and cell wall fragments, in comparison with the activities detected in the medium with only glucose or only cell wall fragments (Fig. 3C). The yeast CFU was determined in each growing medium during 4 days. The *C. oleophila* growth rate was similar in media supplemented with different carbon sources (data not shown).

Detection of exo-β-1,3-glucanase activity of *C. oleophila* in surface wounds

To determine the ability of *C. oleophila* to secrete exo-β-1,3-glucanase in vivo, surface wounds were treated with yeast cells. As shown in Fig. 4, higher levels of exo-β-1,3-glucanase activity were found in extracts of wound tissue treated with *C. oleophila* than in the water-treated control wounds.

To determine whether the glucanase was of yeast or fruit origin, gel activity was used. Detection of exo-β-1,3-glucanase activity on the activity gel revealed one band from wounded sites that had been treated with

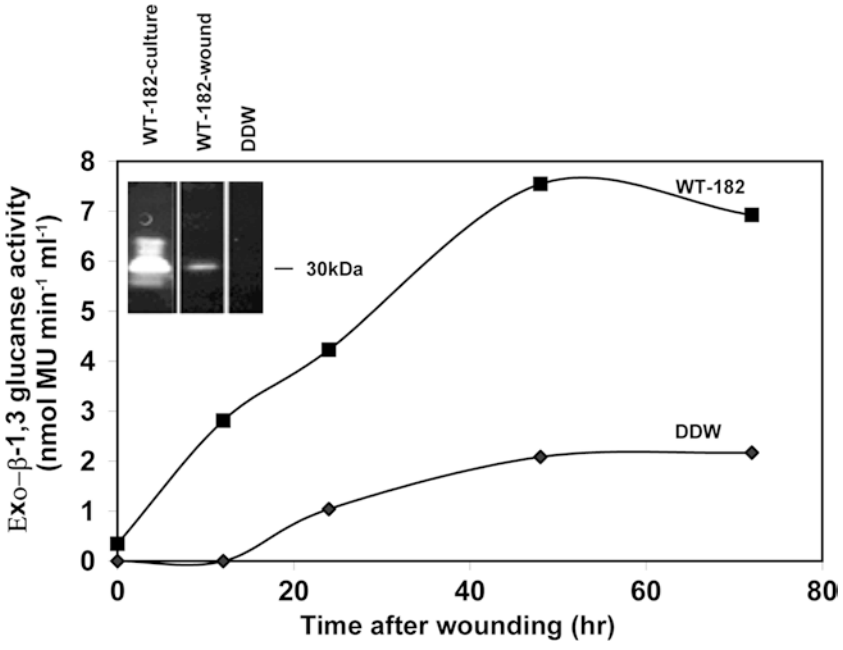

Fig. 4 In vivo detection of exo-β-1,3-glucanase activity from *C. oleophila*. Wound sites of grapefruit were treated with 30 µl of distilled water (*DDW*; control) or 30 µl of a suspension of *C. oleophila* (*WT-182*; 10^8 cells ml^{-1}) and were removed with a cork borer at 0, 12, 24, 48 and 72 h after treatment. Tissues were homogenized in 50 mM sodium acetate buffer (pH 5.0) and centrifuged and the supernatant was collected and assayed for exo-β-1,3-glucanase activity. Assays were performed in triplicate and the experiment was repeated four times. *Insert*: Activity gel profiles of exo-β-1,3-glucanase extracted from wound sites (*wound*) were compared with profiles of the enzyme secreted into the growth medium (*culture*). *hr* Hours

strain 182. This band migrated to the same position as the band of partially purified exo-β-1,3-glucanase obtained from culture filtrate of the yeast (Fig. 4, insert).

In addition, the ability of *C. oleophila* to secrete chitinase and protease into the wound sites was tested. Chitinase activity was detected immediately after wounding and it persisted at the same level. However, no significant difference was found between extracts of wound tissue treated with *C. oleophila* and those of water-treated tissue. Protease activity was hardly detected in the extracts of wound tissue, probably due to the low level of secretion in the samples taken (data not shown).

Inhibitory activity of lytic enzyme secreted by *C. oleophila* in vitro

The inhibitory activities of protein-rich preparations of culture filtrate of *C. oleophila* wild-type (WT-182) and transformants (overexpression T-201, knockout T-789) were tested against spore germination and germ-tube elongation of *P. digitatum*. At a protein concentration of 50 µg ml^{-1}, a 50% inhibition of spore germination was observed in the presence of culture filtrates from both the wild-type and T-201, whereas a similar inhibition percentage was achieved with T-789 only at a protein concentration of 150 µg ml^{-1} (Fig. 5A). Inhibition of germ tube elongation was also observed after enzyme solution treatment (Fig. 5B). The knockout transformant (T-789) was less effective in the inhibition of tube elongation than the overexpression transformant (T-201) and the wild type (WT-182). In addition, at high concentrations of protein, we observed morphological changes in *P. digitatum*, such as leakage of cytoplasm and cell swelling (data not shown).

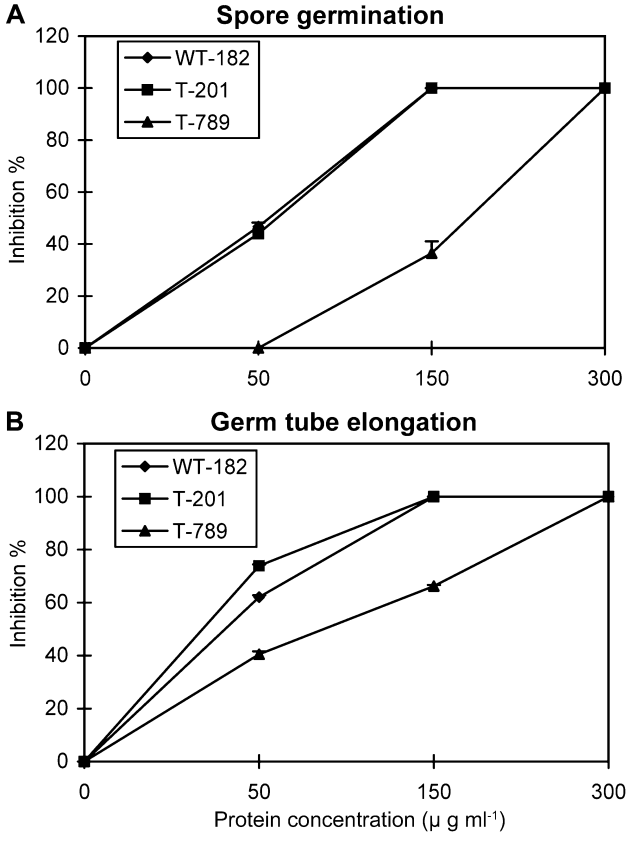

Fig. 5A, B In vitro inhibitory activity of lytic enzyme secreted by *C. oleophila*. The effect of lytic enzymes secreted by the *C. oleophila* wild type and two transformants was studied on spore germination (**A**) and germ tube elongation (**B**) of *P. digitatum*. *Bars* represent the standard error of the mean. Each treatment consisted of three replicates and each experiment was repeated three times

Biocontrol activity of *C. oleophila* transformants

To evaluate the biocontrol activity of the *C. oleophila* transformants (T-789, T-201) against *P. digitatum*, surface wounds of grapefruit were treated with various concentrations of yeast cell suspension (Fig. 6). At concentrations of 10^7 cells ml^{-1} and 10^8 cells ml^{-1}, no differences in biocontrol activity between T-201, T-789 and WT-182 were observed: at these concentrations both transformants and WT-182 effectively reduced the percentage of infected wounds from approximately 100% in the control treatment to about 10% in the yeast-treated wounds. At 10^6 cells ml^{-1}, the knockout transformant (T-789) provided less protection than the wild type and the overexpression transformant (T-201). This reduction in activity was, however, not statistically significant.

Discussion

The results of this study provide evidence that, during its growth, the yeast antagonist *C. oleophila* secretes three enzymes that are thought to be involved in the degradation of fungal cell walls: exo-β-1,3-glucanase, chitinase and protease.

Exo-β-1,3-glucanase and chitinase were produced in the early stages of growth and were maximized within 24–36 h, whereas protease reached its maximum levels only after 6–8 days (Fig. 1A–C). The finding that exo-β-1,3-glucanase and chitinase were produced during the early stages of growth suggests that they have a role in breaking-down complex polysaccharide polymers into small subunits, which can then be consumed as a carbon source by the yeast cells. This finding may have important implications regarding the possible involvement of these enzymes in the biocontrol activity of the yeast antagonist against postharvest pathogens. The availability of nutrients is crucial for the rapid growth of this yeast and its colonization of surface wounds. In addition, production of these two lytic enzymes may have a direct inhibitory effect on the pathogen. The production of protease in the later stages of growth may have a supporting role in achieving the full degradation of the substrates used by this yeast. This role in the biocontrol mechanism may, however, be secondary.

Production of exo-β-1,3-glucanase, chitinase and protease by *C. oleophila* was stimulated by the presence of cell wall fragments of *P. digitatum* in the growth medium, in addition to glucose (Fig. 3A–C). When *C. oleophila* was grown on medium containing only cell wall fragments as the sole carbon source, exo-β-1, 3-glucanase activity was very low compared with that when glucose was present alone, whereas the chitinase and protease activities were not significantly affected by the type of the carbon source in the growth medium. These findings are in contrast with those obtained with *Pichia guilliermondii* and *P. anomala*, which were found to produce higher levels of exo-β-1,3-glucanase when grown in media supplemented with fungal cell walls than when grown in media containing only glucose (Wisnieswski et al. 1991). We suggest that the exo-β-1,3-glucanase secreted from *C. oleophila* requires a supplementary carbon source to maintain the glucose-induced levels of enzyme activity. This could have implications on the way to perhaps improve the biocontrol activity of *C. oleophila*. An available carbon source during the first stage of growth could then use the cell wall fragments.

The present study showed that *C. oleophila* is capable of secreting exo-β-1,3-glucanase into wound sites on fruit, as was confirmed by comparing the molecular masses of the enzymes extracted from the wound tissue and from the culture filtrate on activity gel (Fig. 4). The fact that we detected chitinase activity immediately after wounding and found no difference between wounds treated with *C. oleophila* and those treated with water indicates that we actually measured chitinase activity that originated from the fruit. Previously, it was reported

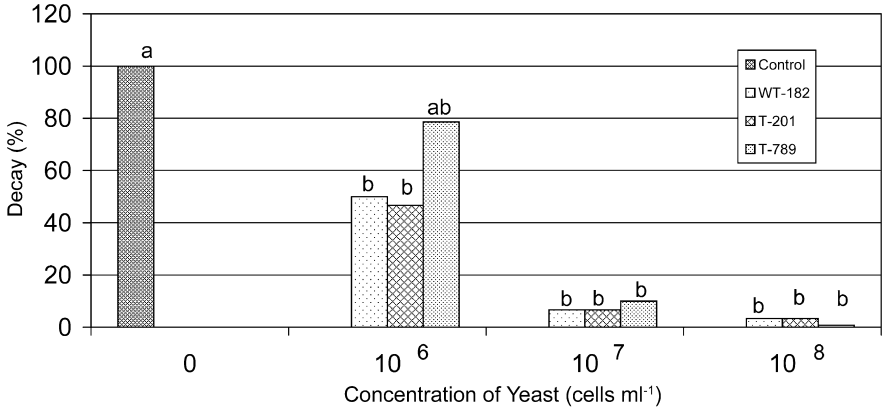

Fig. 6 In vivo biocontrol activity of *C. oleophila*. Wound sites on grapefruit were treated with 30 µl of distilled water (*Control*) or 30 µl of a suspension (10^6–10^8 cells ml^{-1}) of wild-type *C. oleophila* (*WT-182*), the double-*CoEXG1* transformant (*T-201*), or the knockout-*CoEXG1* transformant (*T-789*). Ten fruits with three wounds each were used per treatment. The percentage of wounds infected was measured from the total of 30 wounds. *Values marked by different letters* are significantly different at $P \leq 0.05$, according to an analysis of variance followed by Duncan's multiple range tests. The experiment was conducted three times and the data were pooled

that wounding induced chitinase activity in fruit (Ippolito et al. 2000).

In vitro, experiments were done in order to evaluate whether the exo-β-1,3-glucanase of *C. oleophila* had a role in the inhibition of spore germination and germ-tube elongation by *Penicillium digitatum* (Fig. 5A, B). We found that the wild type *C. oleophila* and the overexpression transformant had similar effects on spore germination and germ-tube elongation by *P. digitatum* and both were more inhibitory to the fungus than the knockout transformant. In vivo experiments were also carried out on grapefruit (Fig. 6): at a high concentration of yeast cells, no significant difference in inhibition was observed between untransformed and transformed *C. oleophila* cells, whereas at lower concentrations the knockout transformants appeared to be less effective than the overexpression transformants. These results indicate that the exo-β-1,3-glucanase secreted by *C. oleophila* did indeed contribute to the antagonistic effect of the yeast, but the fact that the overexpression transformant did not significantly enhance the inhibition of fungal growth implies that exo-β-1,3-glucanase may act in synergy with the other lytic enzymes secreted by *C. oleophila* and that these enzymes may actually be involved in the antagonistic activity of this microorganism. This suggestion seems to be supported by the findings of other studies. El-Katatny et al. (2001) showed that both chitinase and β-1,3-glucanase from the newly isolated *Trichoderma harzianum*T24 inhibit the growth of *Sclerotium rolfsii*; and Gacto et al. (2000) propose that the lytic system of *Micromonospora chalcea* appears to require the conjoint action of both proteinase and glucanase activities to lyse the cell walls of intact living yeast cells.

Collectively, the results of the current study demonstrated the ability of the yeast *C. oleophila* to secret different cell wall-degrading enzymes, including exo-β-1,3-glucanase, chitinase and protease. The role of exo-β-1,3-glucanase, however, was studied in more depth using overexpressing and knockout transformants. The evidence provided thus indicated a possible role for exo-β-1,3-glucanase in the mode of action of the yeast biocontrol agent. However, a more comprehensive study is still needed to elucidate the role of the other cell-degrading enzymes in the antagonistic activity of *C. oleophila*.

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